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Gadd45 proteins: Relevance to aging, longevity and age-related pathologies

Alexey A. Moskalev[a,*], **Zeljka Smit-McBride**[b], **Mikhail V. Shaposhnikov**[a], **Ekaterina N. Plyusnina**[a], **Alex Zhavoronkov**[c], **Arie Budovsky**[d], **Robi Tacutu**[d], and **Vadim E. Fraifeld**[d]

[a]Group of Molecular Radiobiology and Gerontology, Institute of Biology, Komi Science Center of Russian Academy of Sciences, Syktyvkar 167982, Russia

[b]Department of Ophthalmology and Vision Science, School of Medicine, University of California at Davis, Davis, CA 95616, USA

[c]Medical-Biological Faculty, Russian State Medical University, Moscow 119296, Russia

[d]The Shraga Segal Department of Microbiology and Immunology, Center for Multidisciplinary Research on Aging, Ben-Gurion University of the Negev, Beer Sheva 84105, Israel

Abstract

The Gadd45 proteins have been intensively studied, in view of their important role in key cellular processes. Indeed, the Gadd45 proteins stand at the crossroad of the cell fates by controlling the balance between cell (DNA) repair, eliminating (apoptosis) or preventing the expansion of potentially dangerous cells (cell cycle arrest, cellular senescence), and maintaining the stem cell pool. However, the biogerontological aspects have not thus far received sufficient attention. Here we analyzed the pathways and modes of action by which Gadd45 members are involved in aging, longevity and age-related diseases. Because of their pleiotropic action, a decreased inducibility of Gadd45 members may have far-reaching consequences including genome instability, accumulation of DNA damage, and disorders in cellular homeostasis – all of which may eventually contribute to the aging process and age-related disorders (promotion of tumorigenesis, immune disorders, insulin resistance and reduced responsiveness to stress). Most recently, the dGadd45 gene has been identified as a longevity regulator in Drosophila. Although further wide-scale research is warranted, it is becoming increasingly clear that Gadd45s are highly relevant to aging, age-related diseases (ARDs) and to the control of life span, suggesting them as potential therapeutic targets in ARDs and pro-longevity interventions.

Keywords

Gadd45 genes and proteins; Regulatory networks; Aging; Age-related diseases; Longevity

1. Introduction

A decreased ability to cope with stress is one of the hallmarks of aging. Resistance to stress is one of the important determinants of animal survival and longevity (Jazwinski, 1998). Indeed, within a wide variety of species, individuals or strains with longer life spans

*Corresponding author: Tel.: +7 8212 430 650; fax: +7 8212 240 163. amoskalev@ib.komisc.ru, amoskalev@list.ru (A.A. Moskalev).
All authors contributed equally to the article.

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generally demonstrate a higher resistance to environmental and/or physiological stress and *vice versa* (Sohal et al., 1990; Lithgow et al., 1995; Murakami and Johnson, 1996; Lin et al., 1998; Guarente and Kenyon, 2000; Fabrizio et al., 2001; Landis et al., 2003; Brown-Borg, 2006; Masse et al., 2008; Ungvari et al., 2008; Labinskyy et al., 2009; Perez et al., 2009; Amrit et al., 2010; Slack et al., 2010). For example, selection for stress resistance in Drosophila increases its life span (Rose et al., 1992; Harshman et al., 1999). The ability to resist oxidative stress was used as a major criterion for identifying long-lived worm mutants in a genome-wide scale RNAi screen for new longevity regulators (Kim and Sun, 2007). Mutations which contribute to a longer life span in yeast (*Saccharomyces cerevisiae*), worms (*Caenorhabditis elegans*), flies (*Drosophila melanogaster*), and mice (*Mus musculus*) are generally accompanied by increased resistance to starvation, oxidative stress, and heat shock (Migliaccio et al., 1999; Fabrizio et al., 2001; Johnson et al., 2001; Longo, 2003; Rea et al., 2005; Perez et al., 2009). On the other hand, the short-lived mutants of various model organisms show a reduced capacity to cope with unfavorable environmental insults (Vermeulen et al., 2005). In many cases, overexpression of stress-related proteins (e.g., sirtuins, FOXO, HSP70, HSP22, superoxide dismutase, catalase) in certain tissues results in a longer life span of model organisms (Saunders and Verdin, 2009). Reduced resistance to stress was suggested to be both a cause and an effect of aging (Pandolf, 1997; Ikeyama et al., 2002). Further strengthening the links between stress and longevity is the observation that moderate exposures to harmful factors such as thermal or oxidative stress, ionizing radiation, and hypergravity can stimulate protective mechanisms and eventually lead to increased longevity ("longevity hormesis") (Crawford and Davies, 1994; Moskalev, 2007; Moskalev et al., 2009; Rattan et al., 2009; Saunders and Verdin, 2009; Rattan, 2010).

Among the stress-associated genes, the members of the Gadd45 family play an important role in the integration of cellular response to a wide variety of stressors in mammals (Liebermann and Hoffman, 1998; Zhang et al., 1999; Fornace et al., 2002). At basal conditions, the expression levels of the Gadd45 family members are relatively low, but they are highly inducible upon a wide plethora of stressful stimuli, both physiological and environmental. This is summarized in Table 1. Of note, the median half-life of the *Gadd45* mRNA is unusually short (less than 1 h), suggesting a regulatory rather than metabolic function for Gadd45 proteins (Sharova et al., 2009). Genotoxic and oxidative stress can rapidly induce their transcription (Fornace et al., 1989; Papathanasiou et al., 1991) and increase the mRNA stability of the *Gadd45α* (Jackman et al., 1994). Expression of *Gadd45α* is stimulated by physical stress such as UV-radiation (Fornace et al., 1988), X-rays (Papathanasiou et al., 1991), γ-radiation (Papathanasiou et al., 1991; Gajdusek et al., 2001), low-frequency electromagnetic fields (Nikolova et al., 2005), as well as by hypoxia (Price and Calderwood, 1992), peroxynitrite free radicals (Oh-Hashi et al., 2001), hyperosmotic (Drews-Elger et al., 2009) and oncogenic stress (Bulavin et al., 2003), low pH (Duggan et al., 2006), arachidonic acid metabolite and growth inhibitor Delta12-prostaglandin J2 (Ohtani-Fujita et al., 1998), a component of the C5b-9 complement system (Pippin et al., 2003), xenobiotics such as arsenic (Bower et al., 2006), Cr(VI) compounds (Ceryak et al., 2004), cisplatinum (DeHaan et al., 2001), alkylating agent methyl methane sulfonate (Zhang et al., 2001), ethanol (Ji et al., 2005), cigarette smoke condensate (Fields et al., 2005), and many other soil, air, and water pollutants (Sen et al., 2007). Different inducers of oxidative stress including sodium arsenite, carbon tetrachloride, bacterial lipopolysaccharides, inflammatory cytokines IL-6, IL-12 and IL-18, pro-apoptotic cytokines TGFβ and TNFα stimulate the expression of *Gadd45β* (Selvakumaran et al., 1994; De Smaele et al., 2001; Lu et al., 2001; Yang et al., 2001; Takekawa et al., 2002; Amanullah et al., 2003; Yoo et al., 2003; Zhang et al., 2005). *Gadd45γ* is induced by bacterial lipopolysaccharides that trigger inflammation, and also in response to pro-inflammatory cytokines IL-2 and IL-6 (Zhang et al., 1999; Altemeier et al., 2005).

The Gadd45 proteins are implicated in many basic processes shown to be intimately linked to aging and age-related diseases (ARDs), including DNA repair (Smith et al., 1994; Vairapandi et al., 1996), maintaining genome stability (Hollander et al., 1999), epigenetic regulation (Muñoz-Najar and Sedivy, 2011), cell cycle arrest (Beadling et al., 1993; Hollander et al., 1999; Wang et al., 1999; Zhang et al., 1999), cellular senescence (Tront et al., 2006), apoptosis (Harkin et al., 1999; Vairapandi et al., 2000; Azam et al., 2001; Takekawa et al., 2002; Yoo et al., 2003), cell survival (Smith et al., 1996; De Smaele et al., 2001; Zazzeroni et al., 2003; Papa et al., 2004; Gupta et al., 2005), inflammatory responses and immunity (Lu et al., 2001, 2004; Yang et al., 2001), and embryogenesis (Hoffman and Liebermann, 2009). Recently, we have shown for the first time that overexpression of the *dGadd45* gene in the nervous system of Drosophila leads to the extension of the maximum life span, without compromising such life quality factors as physical activity and female fertility (Plyusnina et al., 2011). Given that longevity-associated genes are also deeply involved in major ARDs and aging-associated conditions (Budovsky et al., 2009; Wolfson et al., 2009; Tacutu et al., 2010), studying the mammalian Gadd45 family may provide potential therapeutic targets for combating ARDs and promoting longevity.

Our knowledge regarding the impact of Gadd45 proteins on ARDs and aging-associated conditions has not yet been systematically reviewed. With this in mind, we have undertaken extensive analysis of relevant literature and using the systems biology tools highlighted the Gadd45-associated pathways relevant to aging, longevity and ARDs.

2. Gadd45 family: structure, partners, and evolutionary conservation

The Gadd45 abbreviated name stands for Growth Arrest and DNA Damage-inducible (Fornace et al., 1989; Papathanasiou et al., 1991). Currently, three mammalian genes of the *Gadd45* family, *Gadd45α*, *Gadd45β* and *Gadd45γ* are known. The *Gadd45α* gene was discovered as a rapidly induced transcript in response to UV-irradiation of the Chinese hamster ovary cells (Fornace et al., 1988). Another gene of the Gadd45 family, *Gadd45β* (originally designated as *MyD118*) was cloned as a primary response gene in myeloid differentiation (Abdollahi et al., 1991). *Gadd45γ* was found in mice as the ortholog of the human *CR6* gene encoding an acute phase response protein expressed upon the interleukin-2 stimulation (Zhang et al., 1999).

In humans, the *Gadd45* genes are localized to three different chromosomes (chr 1, 19, and 9 for *Gadd45α*, *Gadd45β*, and *Gadd45γ*, respectively). Of four *Gadd45α* transcripts, only two are protein-coding. *Gadd45β* encodes for one protein and *Gadd45γ* produces three transcripts of which two are protein-coding. Distinct isoforms for *Gadd45α* and *Gadd45γ* are produced through alternative splicing (Flicek et al., 2011). It has not yet been established whether there is any functional difference between these isoforms.

The proteins of the Gadd45 family are relatively small (about 18 kDa), acidic (pH 4.0–4.2), with a high degree of homology among them (55–57% of identical amino acid sequence), and predominantly localized to the nucleus (Abdollahi et al., 1991; Zhang et al., 1999; Vairapandi et al., 2002). Gadd45 proteins are able to form homo- and hetero-dimers with other Gadd45 members, as well as oligomers, dimers being the predominant form (Kovalsky et al., 2001; Schrag et al., 2008). Each of the *Gadd45* genes is expressed in multiple types of mammalian tissues including heart, brain, spleen, lungs, liver, skeletal muscles, kidneys, and testes (Zhang et al., 1999). Specifically, *Gadd45γ* is highly expressed in placenta.

Although the Gadd45 family members have much in common, they still differ in their regulation and function (Liebermann and Hoffman, 2003). This could be attributed to some diversity in the spectrum of binding transcription factors, 3D structure [resolved thus far for Gadd45α (Sánchez et al., 2008) and Gadd45γ (Schrag et al., 2008)], and in interacting

partners, described in Fig. 1. Until now, several regulatory elements have been found in the promoters of *Gadd45* genes, including binding sites for transcription factors Egr1 (early growth response 1), WT1 (Wilms tumor 1), Oct-1 (POU2F1, POU class 2 homeobox 1), NFYA (nuclear transcription factor Y, alpha; a subunit of a trimeric complex which also contains the NFYB and NFYC), and NF-κB (Zhan et al., 1998; Jin et al., 2001; Fan et al., 2002; Daino et al., 2003). In addition, there are binding motifs in the third intron of *Gadd45α* for p53, AP-1 (a heterodimeric complex composed of c-Fos, c-Jun, ATF and JDP proteins), NF-AT, HNF-3 and KLF (Jin et al., 2001; Daino et al., 2003, 2006).

Gadd45 proteins contain the highly conserved L7Ae/L30e/S12e/Gadd45 domain, found in archaea, eubacteria and eukaryota. This domain confers the RNA binding properties and characteristics of a ribonucleoprotein particle to Gadd45α (Sytnikova et al., 2011). Our analysis using the InParanoid database (Ostlund et al., 2010) showed that Gadd45 orthologs first appeared in mollusks, and was also found in anemones, polychaete worms, insects, fish, amphibians and mammals. The number of Gadd45 proteins in each species varies from one in lower organisms to 5–6 in fish, and decreases to 2–3 in amphibians and mammals. This indicates that the Gadd45 family is relatively "young" and has undergone duplications and deletions in the course of evolution.

3. Gadd45 involvement in stress responses and maintaining the cellular homeostasis

3.1. GADD45 in DNA repair and epigenetic regulation

As mentioned above (see Table 1), the genotoxic stress of various natures could induce the transcriptional activity of *Gadd45* genes, thus evoking Gadd45-mediated DNA repair. Several tumor suppressors involved in DNA repair and the FOXO transcription factors are particularly important in linking the stressful signals with Gadd45 repair activity. It is important to stress that since Gadd45 proteins are non-enzymatic, all their actions are realized through the interactions with either their protein partners or modulation of DNA/RNA accessibility to other proteins.

One of the central DNA damage sensors – p53, a tumor suppressor protein which regulates cell cycle, DNA repair and apoptosis, is involved in the genotoxic stress-induced upregulation of Gadd45α (Kastan et al., 1992; Guillouf et al., 1995; Vairapandi et al., 1996; el-Deiry, 1998). For example, upon benzene treatment, the mouse bone marrow $p53^{+/+}$ cells express more Gadd45α than the $p53^{+/-}$cells (Boley et al., 2002). Further supporting the role of p53 in the regulation of Gadd45 expression is that the Gadd45-mediated cell cycle arrest is not observed in the Li-Fraumeni fibroblast culture bearing p53 gene mutation (Wang et al., 1999) (see also Section 4). Another tumor suppressor protein p33 (ING1) which along with p53 stimulates DNA repair, was also shown to interact with Gadd45α (Cheung et al., 2001). The member of p53 family p73 protein is capable of stimulating *Gadd45α* expression as well (De Laurenzi and Melino, 2000). The tumor suppressor BRCA1 was shown to mediate the Gadd45α upregulation triggered by ionizing radiation (MacLachlan et al., 2000).

Another important group of regulators that evoke Gadd45-mediated DNA repair activity are the transcription factors of the FOXO family. The FOXO proteins regulate many mammalian genes associated with longevity and resistance to various types of stress including oxidative stress (Sedding, 2008). The *Gadd45* gene promoter contains FOXO-binding motifs and activation of AFX/FOXO4 and FKHRL1/FOXO3A transcription factors upon oxidative stress results in Gadd45 upregulation with subsequent stimulation of DNA repair and blockage of the transition between G2/M cell cycle phases (Furukawa-Hibi et al.,

2002; Tran et al., 2002). In contrast, the disrupted expression of *Gadd45β* caused by the hepatitis C viral infection suppresses the DNA excision repair (Higgs et al., 2010).

While it is not completely clear how Gadd45γ participates in the repair of DNA, there is strong evidence indicating an important role of Gadd45α and Gadd45β in DNA excision repair through their interaction with the proliferating cell nuclear antigen (PCNA), one of the key molecules in cell cycle regulation (Smith et al., 1994, 2000) and in the stabilization of DNA replication and repair machinery (Essers et al., 2005). Specifically, PCNA stabilizes binding of DNA polymerase delta (involved in resynthesis of excised damaged DNA strands during DNA repair) to DNA. The PCNA proteins encircles DNA and forms a sliding clamp that holds the DNA replication and repair complexes close to DNA (Ellison and Stillman, 2003).

Strongly supporting the role of Gadd45 in DNA repair are studies on the *Gadd45a*$^{-/-}$ mice which were found to exhibit genomic instability, reduced nucleotide excision repair, an increased level of mutations and high susceptibility to chemically induced tumorigenesis (Hollander et al., 1999, 2001) (this mouse model is also discussed in Section 4.1. The list with description of phenotypic features of *Gadd45* knockouts is presented in Table 2).

Involvement of Gadd45 in DNA repair is directly connected to its ability to facilitate the physical approach of several proteins to DNA. This allows epigenetic activation of genes through the repair-mediated active DNA demethylation (Barreto et al., 2007; Rai et al., 2008; Sen et al., 2010; Cortellino et al., 2011). The key role of Gadd45 in linking DNA repair and epigenetic regulation is highlighted by the coupling of cytosine deamination with base and nucleotide excision repair (Ma et al., 2009; Schmitz et al., 2009). This coupling results in active DNA demethylation which is partially attributed to the ability of Gadd45 proteins to bind histones and modify accessibility of DNA on damaged chromatin (Carrier et al., 1999). Gadd45 was also shown to participate in chromatin decondensation (Carrier et al., 1999; Ma et al., 2009).

While the exact mechanisms of Gadd45-mediated demethylation remain unclear, they could involve nucleotide excision repair (NER) associated with the endonuclease activity of xeroderma pigmentosum group G (XPG). Specifically, 5-methylcytosine containing nucleotides could be recognized and removed through Gadd45–XPG complex, ultimately resulting in the demethylation of CpG dinucleotides (Ma et al., 2009; Schmitz et al., 2009; Le May et al., 2010; Schäfer et al., 2010). Of note, both Gadd45 and XPG are also involved in base excision repair (BER), which could be another DNA repair mechanism associated with removal of methylated DNA (Jung et al., 2007).

Recently, Gadd45α was shown to bind RNA, forming ribonucleoprotein particles (Sytnikova et al., 2011). Gadd45 was detected inside nuclear speckles which are sites of active transcription, RNA splicing and processing. Thus, Gadd45 could exert its epigenetic effects both through active DNA demethylation, chromatin remodeling and post-transcriptional RNA regulation, which are intimately linked to Gadd45-mediated DNA repair.

3.2. Gadd45 in cell cycle arrest and cellular senescence

The damaged cell often enters a cell cycle arrest in order to allow time for DNA repair. Transient delays of cell division occur to prevent both replication of a damaged DNA template and segregation of damaged chromosomes. If the repair is successful, the cell cycle progresses. Otherwise, a non-reparable damage results in the prolonged or permanent cell cycle arrest (cellular senescence) or in apoptosis. Gadd45 is essential for a DNA damage-induced G_2/M cell cycle arrest in both human and mouse fibroblasts (Wang et al., 1999).

Microinjection of the *Gadd45α* expression vector into human primary fibroblasts arrests the cells in G2/M phase (Wang et al., 1999). The same Gadd45γ-dependent effect was achieved by withdrawal of growth stimuli (serum deprivation) in HeLa cells (Zhang et al., 2001).

Ectopic expression of Gadd45α, Gadd45β or Gadd45γ in cancer cells (M1 human myeloblastic leukemia and H1299 lung carcinoma) leads to the accumulation of cells arrested in the G1 phase (Zhang et al., 2001). In contrast to the cells arrested in the G2/M phase, the above G1 arrested cells later underwent apoptosis (Zhang et al., 2001).

Gadd45 proteins achieve cycle arrest by physically interacting with several proteins including protein kinase cell division cycle 2 (Cdc2), cyclin B1, and p53-inducing proteins such as PCNA and cyclin-dependent kinase inhibitor p21 (Liebermann and Hoffman, 2003). The interaction of Gadd45 with the Cdc2/cyclin B1 kinase complex is responsible for the G2/M cell cycle arrest through dissociation of the kinase complex (Zhan et al., 1999; Zhang et al., 1999; Vairapandi et al., 2002) and inhibition of Cdc2 kinase activity. The interaction of Gadd45 with p21 may induce the G1 arrest (Xiong et al., 1993; Zhang et al., 1993; el-Deiry, 1998).

One of the possible outcomes of DNA damage is the commitment of cells to cellular senescence, an irreversible cell cycle arrest in the G1 phase with subsequent unresponsiveness to growth factors. Gadd45 proteins are intimately involved in this process. Cellular senescence in cultures of human fibroblasts is associated with a p53-dependent induction of *Gadd45α* (Jackson and Pereira-Smith, 2006). Of note, in senescent diploid fibroblasts, p53 predominantly binds to the promoters of p21 and *Gadd45,* the cell growth arrest genes, rather than to other p53 targets involved in regulation of apoptosis (such as *TNFRSF10B*, *TNFRSF6*, and *PUMA*) (Jackson and Pereira-Smith, 2006). This may in part explain the resistance of senescent cells to apoptosis.

Gadd45α is involved in cellular senescence induced by diverse factors (Hollander et al., 1999; Bulavin et al., 2003). For example, a significant increase in *Gadd45α* expression was observed upon stress-induced cellular senescence triggered by H_2O_2 (Duan et al., 2005). This process was also characterized by an increased expression of p21, involving mitochondrial dysfunction and generation of reactive oxygen species through the Gadd45/p38 MAPK/GRB2/TGFBR2/TGFβ signaling pathway, induced by prolonged activation of p21 as a result of the DNA damage (Passos et al., 2010).

3.3. Dual role of Gadd45 in apoptosis and cell survival

As mentioned above (Section 3.2), the excessive DNA damage beyond a certain threshold may trigger apoptosis. In the case of irreparable damage, Gadd45 proteins exert a pro-apoptotic function. For example, the Gadd45 proteins mediate the endoplasmic reticulum stress-induced apoptosis in mouse liver cells (Ji et al., 2005). Another example includes the ectopic expression of Gadd45 in human leukemic cells or in mouse hepatocytes, which triggers apoptosis via the TGFβ/MEKK4/p38/JNK pathway, whereas blockage of the *Gadd45β* expression suppresses the apoptosis induced by TGFβ (Selvakumaran et al., 1994; Yoo et al., 2003).

However, in the case of moderate DNA damage, the Gadd45α and Gadd45β proteins act as anti-apoptotic agents and, for example, increase hematopoietic cell survival under UV-irradiation or treatment with certain chemotherapeutic drugs (Gupta et al., 2005). Bone marrow cells obtained from *Gadd45α*$^{-/-}$ and *Gadd45β*$^{-/-}$ mice show an impaired ability for differentiation and increased sensitivity to the induction of apoptosis after being stimulated by cytokines (Gupta et al., 2006). Anti-apoptotic function of *Gadd45* is realized through two different ways: via activation of the p38/NF-κB anti-apoptotic pathway by Gadd45α (Gupta

et al., 2006) and inhibition of the MKK7/JNK pro-apoptotic pathway by Gadd45β (Papa et al., 2004; Tornatore et al., 2008). Consistent with the idea that interactions of Gadd45 proteins with PCNA may promote cell survival by enhancing DNA repair is the observation that Gadd45/PCNA complexes inhibit apoptosis (Vairapandi et al., 2000; Azam et al., 2001).

3.4. GADD45 in cell differentiation and maintenance of the quiescent stem cell pool

Gadd45 family members play an important role in cell differentiation, both during embryonic development and in the mature organism. Moreover, there is evidence that the expression of Gadd45 members may be cell type-specific, as was recently shown by Kaufmann et al. (2011) in mouse embryos.

In many cases, the cell differentiation is preceded by cell cycle arrest in G1 phase and is consistently accompanied by an increased expression of Gadd45 proteins. For example, Gadd45γ is a primary transcription target of Ascl1, a transcription factor involved in neuronal differentiation (Huang et al., 2010). The differentiation-promoting role of XGadd45γ during neurogenesis was also shown in *Xenopus* embryos (de la Calle-Mustienes et al., 2002).

In another study on zebrafish, it was shown that Gadd45β expression in the anterior presomitic mesoderm is required for somite segmentation (Kawahara et al., 2005). Gadd45β was also found to play an important role in the differentiation of chondrocytes. It was identified as an early response gene induced by the bone morphohenic protein BMP-2 via a Smad1/Runx2-dependent pathway (Ijiri et al., 2005). In turn, it was shown that during the neural development in chicken embryo, an increased expression of Gadd45s induced by retinoic acid, promotes proteasomal degradation of Smad1 (Sheng et al., 2010). Based on this, it is attractive to suggest the existence of a Gadd45-Smad1 regulatory loop.

Treatment of myeloid cells with IL-3, GM-CSF, G-CSF, or M-CSF cytokines known as important players in myeloid differentiation rapidly induces the expression of all three *Gadd45* genes (Zhang et al., 1999). Furthermore, induction of the *Gadd45* genes in response to various cytokines at the initial stage of myeloid differentiation suggests that Gadd45 proteins play a role in hematopoiesis (Abdollahi et al., 1991).

The Gadd45 molecules effect not only differentiation but also the maintenance of a pool of myeloid quiescent stem cells. Though the mechanisms underlying these effects are still unknown, *Gadd45α* or *β* deletion was shown to either directly suppress the quiescent stem cell population or lower the survival rate of progenitor cells, thus leading to the depletion of the stem cell compartment (Hoffman and Liebermann, 2007). *Gadd45* deletion also affects clonogenic potential of myeloid progenitor cells (Hoffman and Liebermann, 2007).

A possible hint to the regulatory mechanism could be the observation that the Nucleus accumbens-1 (Nac1, or NAC-1) protein which is important for self-regeneration and pluripotency of embryonic stem cells, negatively regulates the expression of Gadd45γ-interacting protein 1 (Gadd45γ-ip1), thus preventing its suppressive activity towards Gadd45γ (Jinawath et al., 2009). All the accumulated data indicate that Gadd45 proteins are important for promoting cell differentiation and maintaining the stem cell pool.

3.5. Gadd45 in immunity

Apart from their activities at the cellular level, the Gadd45 proteins also contribute to organismal survival by modulating the immune responses. The Gadd45 proteins stimulate proliferation of T helper 1 (Th1) cells which belong to the acquired branch of the immune system (Yang et al., 2001). Besides, Gadd45β and Gadd45γ induce production of the IFNγ by Th1 cells, thus further boosting the activation of the immune system (Yang et al., 2001).

The essential role of Gadd45 in elevating the level of IFNγ is clearly evidenced by the disruption of this process in *Gadd45γ* - (Lu et al., 2001) and *Gadd45β*-deficient mice (Ju et al., 2009). Also, the study on *Gadd45β* and *Gadd45β*/*Gadd45γ* double knockout mice revealed the crucial importance of Gadd45 members, and particularly Gadd45β, in Th1-mediated anti-tumor immune responses (Ju et al., 2009) and in regulating autoimmunity (Liu et al., 2005). *Gadd45β* has recently been identified as an immunological tolerance (anergy)-associated gene (Safford et al., 2005). Activation of its transcription by the Notch signaling associated protein Deltex 1 (DTX1) results in induction of the T cell-mediated anergy. On the other hand, deletion of DTX1 decreases (but not abolishes) the expression of *Gadd45β* and promotes the maturation of T cells, giving rise to enhanced production of antibodies and increased inflammation (Hsiao et al., 2009). Collectively, Gadd45 contributes to enhancing the immune surveillance of tumors and elimination of cells that might display autoimmune behavior.

3.6. Gadd45 in stress signaling

The involvement of Gadd45 in stress signaling is partly discussed in previous sections. This issue was intensively reviewed (for example, see: Gao et al., 2009; Yang et al., 2009) and its detailed discussion is beyond the scope of this paper. Here we would like to emphasize that the involvement of Gadd45 proteins in various cell activities (see Sections 3.1–3.5) depends on different combinations of interactions with their partners (see Figs. 1 and 2). Nevertheless, the common basis of action for all three Gadd45 proteins lies in connecting an upstream sensor module (MAPK-associated cascade) to the downstream transcription regulatory module (NF-κB molecule). In fact, the MAPK/Gadd45/NF-κB axis responds to a variety of extracellular stimuli, converting them to intracellular responses. In most cases, in response to stress, the Gadd45 proteins stimulate the p38/JNK MAPK signaling pathway, thus increasing the sensitivity of cells to growth arrest, apoptosis or survival (Takekawa and Saito, 1998; Harkin et al., 1999; Lu et al., 2001; Hildesheim et al., 2002; Yoo et al., 2003). For example, Gadd45γ and Gadd45β bind to MEK kinase 4 (MEKK4) and promote phosphorylation and activation of the p38 and JNK MAP kinases by MEKK4 (Takekawa and Saito, 1998). The effect of Gadd45 on stress kinases is cell type-specific: activation of p38 and JUNK MAPKs by Gadd45 is associated with apoptosis in endothelial and epithelial cells (Harkin et al., 1999; Hildesheim et al., 2002), whereas it increases survival of hematopoietic cells (Platanias, 2003). The opposite effect of Gadd45β on JNK activity was observed in other types of cells: induction of G*add45β* by NF-κB downregulates pro-apoptotic JNK signaling in mouse embryonic fibroblasts (De Smaele et al., 2001) and in hepatocytes during liver regenerations after partial hepatectomy (Papa et al., 2008). Of note, Gadd45 proteins not only modulate the activity of stress kinases, but the expression of Gadd45 is also under the control from p38 and JUNK MAPKs. The latter is supported by the observation that the specific inhibitor of p38 MAPK SB202190 suppresses the expression of all three *Gadd45* genes (Oh-Hashi et al., 2001). Thus, it seems plausible that Gadd45 and stress kinases form a feedback regulatory loop.

Gadd45 is directly involved in modulating the cell protective effect of NF-κB, well known for its anti-apoptotic activity along with reduction in free radical formation (Zhang et al., 2005). Gadd45α stimulates degradation of the NF-κB inhibitor IκB, followed by relocalization of NF-κB into the nucleus and activation of its target genes (Gupta et al., 2006). Importantly, NF-κB can stimulate the expression of *Gadd45* and simultaneously acts as its regulatory target, thus (till a certain threshold) creating a positive feedback regulatory loop. However, a constitutive activation of NF-κB decreases the expression of *Gadd45α* and *Gadd45β* (Zerbini et al., 2004).

As previously mentioned (Sections 3.1–3.5), Gadd45 family members are important regulators in several other pathways (p53, FOXO, TGFβ, Wnt, etc.), in addition to stress

MAPKs/NF-κB pathway. All these pathways substantially overlap, and to a great extent shape the fate of the stressed cell.

4. Gadd45 in age-related pathologies and aging-associated conditions

4.1. Cancer

Aging is the major risk factor for cancer, and incidence of breast, prostate, colon, lung, stomach, bladder and skin cancer dramatically increases with age (Hoeijmakers, 2009). Aging-associated conditions such as oxidative stress, chronic inflammation and immunosenescence predispose to tumorigenesis (Chung et al., 2009).

The anti-tumor activity of Gadd45 was shown both *in vitro* and *in vivo* studies. For example, ectopic expression of Gadd45 members blocks cell growth by arresting the cells at the G2/M phase (Zhu et al., 2009) and/or induces apoptosis in several human tumor cell lines such as M1 myeloblastic leukemia, H1299 lung carcinoma, HeLa cervical cancer, RKO colon carcinoma, and in non-transformed NIH3T3 mouse embryonic fibroblasts (Zhan et al., 1994; Vairapandi et al., 1996; Zhang et al., 2001; Sun et al., 2003; Jiang and Wang, 2004; Ying et al., 2005). Also, Gadd45 was shown as being involved in fibroblast growth factor-2 (FGF-2)-induced differentiation of SK-N-MC human neuroblastoma cells via promoting the cell cycle arrest in G1/G0 phase (Higgins et al., 2009). The anti-cancer activity of *Gadd45α* is strongly supported by studies on the mouse knockout models. Mice with a *Gadd45α* gene deletion show genomic instability and increased sensitivity to carcinogenesis (Hollander et al., 1999; Tront et al., 2006). The *Gadd45α* knockout mice are also more prone to DMBA-induced ovarian tumors, hepatocellular tumors in males, and vascular tumors in both sexes (Hollander et al., 2001). The increased rate of tumorigenesis in these mice could be attributed to a very low efficiency of the thymidine dimer and nucleotide excision repair as already described in previous sections of this work (Hollander et al., 2001; Maeda et al., 2002). Mice with the *Gadd45β* gene knockout are more susceptible to ionizing radiation and chemical carcinogens and display a lower immune response against implanted melanoma cells (Ju et al., 2009). In the *in vivo* model of Ras-overexpressing mice which differ in the levels of Gadd45α expression (Ras/Gadd45α$^{+/+}$, Ras/Gadd45α$^{+/-}$, and Ras/Gadd45α$^{-/-}$), it was shown that Ras-driven breast tumorigenesis is Gadd45α-dependent (Tront et al., 2006). The authors concluded that Gadd45α inhibits the onset and growth of mammary tumors via induction of JNK-activated apoptosis and p38-mediated cellular senescence (Tront et al., 2006). On the other hand, the loss of the *Gadd45α* gene accelerates the mammary tumor growth (Tront et al., 2006). These findings coincide well with the clinical observations that *Gadd45α* is frequently deleted in breast cancer (Hoggard et al., 1995). Furthermore, disruption in the *Gadd45* expression was observed in multiple types of solid and hematopoietic malignancies including nasopharyngeal, breast, lung and prostate cancer, hepatocellular tumors, pituitary adenoma, and lymphoma (Qiu et al., 2003, 2004; Sun et al., 2003; Jiang and Wang, 2004; Ying et al., 2005; Cretu et al., 2009; Na et al., 2010).

The silencing of *Gadd45* in cancers could be greatly attributed to epigenetic changes that typically occur with advanced age. In particular, promoters of many tumor suppressors were found to be hypermethylated in aging, with subsequent transcriptional gene silencing (Muñoz-Najar and Sedivy, 2011). A similar situation was observed in many cancers (Muñoz-Najar and Sedivy, 2011). Thus, this common epigenetic modification could be important in linking cancer and aging. With regard to this notion, the methylation of the *Gadd45γ* promoter was found to be significantly higher in different types of cancer than in normal tissues (Zhang et al., 2010). Nevertheless, treatment of cancer cells with DNA methyltransferase inhibitors can restore *Gadd45β* expression to its level in the non-tumorous cells (Qiu et al., 2004).

One of the mechanisms which could lead to the increased methylation of *Gadd45* in aging may be attributed to a constitutive activation of NF-κB, a quite typical age-related condition. This transcription factor induces the expression of proto-oncogene c-Myc (Zerbini et al., 2004; Zerbini and Libermann, 2005), which binds to the GC-rich sites of the *Gadd45* promoters and significantly reduces the *Gadd45* expression in response to genotoxic stress (Amundson et al., 1998). It seems plausible that c-Myc-mediated repression of *Gadd45* expression occurs through recruitment of DNA methyltransferase Dnmt3a, as was reported for p21 (Brenner et al., 2005). The inhibition of NF-κB in cancer cells leads to the Gadd45α- and γ-dependent induction of apoptosis and reduction in tumor growth (Zerbini et al., 2004), further supporting the role of NF-κB in the regulation of Gadd45 expression. The epigenetic coupling between cell death and the expression levels of Gadd45 is also exemplified by histone deacetylase inhibitors such as trichostatin and butyrate, which induce Gadd45α and Gadd45β expression and cause apoptosis in human carcinomas (Chen et al., 2002; Campanero et al., 2008).

Other examples connecting the Gadd45 expression levels to the induction of apoptosis in cancer cells include several pharmacological agents. A widely known drug Troglitazone which possesses the anti-cancer, anti-diabetic and anti-inflammatory activities inhibits proliferation and induces apoptosis in MCF7 human breast carcinoma cells (Yin et al., 2004). Remarkably, out of the 23 Troglitazone-induced genes, not only had *Gadd45α* the highest expression level, but its knockdown abrogated the Troglitazone-induced apoptosis (Yin et al., 2004). Stimulation of Gadd45 expression by arsenic trioxide (Li et al., 2003) or an anti-tumor flavonoid quercetin (Yoshida et al., 2005) also leads to apoptosis in cancer cells.

Apart from the “canonical” anti-cancer activity of Gadd45 proteins through the induction of apoptosis in cancer cells, Gadd45α was found to be an important negative regulator of two oncogenes commonly over-expressed in epithelial tumors, p63 and β-catenin (Hildesheim et al., 2004). Gadd45α prevents translocation of β-catenin (the effector molecule of Wnt signaling) into the nucleus where it functions as a transcription factor involved in the induction of cell proliferation. Among its targets are also endopeptidases and matrix metalloproteinases (MMP3 and MMP9). These enzymes are important players in the remodeling of extracellular matrix enabling the invasion and metastasis of cancer cells. Thus, not only are Gadd45s involved in killing the cancer cells, but they also suppress cancer progression by inhibiting the cell migration. The latter has recently been substantiated by a study on cancer cells with different migration potentials (Trabucco, 2010). The migration levels of the BL185 murine cell line with the stable shRNA Gadd45α knockdowns were significantly increased versus the parental non-metastasizing line. Furthermore, hepatocellular carcinoma (HCC) cell lines with high migration ability exhibited a reduced expression of *Gadd45α*.

In view of the established role of Wnt signaling and MMPs in epithelial-mesenchymal transition (EMT) (Przybylo and Radisky, 2007), its inhibition may be another putative mechanism by which Gadd45s may exert its tumor suppressor activity. This phenomenon is characterized by the downregulation of epithelial markers and loss of intercellular junctions with subsequent breakage of epithelial polarity, loss of contact inhibition, and acquisition of mesenchymal features that eventually contribute to cancer initiation and progression (Christiansen and Rajasekaran, 2006). This possible aspect of Gadd45 anti-tumor activity has not as yet been directly addressed.

In contrast to its well established anti-cancer role, in some cases, Gadd45α may exert an opposite action, depending on the type of the oncogenic stimuli. For example, while Ras-driven breast tumorigenesis is suppressed by Gadd45α, the Myc-driven breast cancer is

promoted by Gadd45α (Tront et al., 2010). In the case of c-Myc-driven breast carcinogenesis, activation of Gadd45α results in a dramatic decrease in the level of MMP10, an enzyme promoting angiogenesis. Consequently, its inhibition by Gadd45α results in stimulation of tumor vascularization. In the presence of Myc, loss of Gadd45α was accompanied by activation of either apoptosis or cellular senescence, an outcome opposite to that observed in the case of Ras-driven breast tumorigenesis (Tront et al., 2010).

4.2. Other age-related diseases

While most of our knowledge with regard to the involvement of Gadd45 in the age-related pathologies comes from cancer studies, some evidence has been accumulated pointing to its role in other age-related conditions as well. For example, *Gadd45* is highly expressed in neurons of Alzheimer's disease patients and protects the neurons from apoptosis induced by extracellular accumulation of β-amyloid (Torp et al., 1998; Santiard-Baron et al., 1999, 2001). The upregulation of Gadd45 was also observed in the *in vitro* model (human neuroblastoma cells) of dopamine-induced neurotoxicity (Stokes et al., 2002). This observation could be relevant to the suggested role of ROS and reactive quinones, produced during dopamine oxidation, in pathogenesis of both Parkinson's disease and normal brain aging (Stokes et al., 2002). An increased expression of Gadd45 in age-related neurodegeneration is likely a protective mechanism aimed at coping with the neurotoxic stress.

The same could also be the case in another major ARD, atherosclerosis. As was shown in primary cultures of human endothelial cells derived from atherosclerotic aorta or coronary arteries, activation of the lectin-like oxLDL receptor (LOX-1) leads to DNA damage and to a 4-fold increase in *Gadd45* expression (Thum and Borlak, 2008). A comparable increase in *Gadd45* transcription of aortic endothelial cells was also observed in a mouse model of atherosclerosis (ApoE knockout mice fed with a high lipid diet for >4 months) (Thum and Borlak, 2008).

The remarkable induction of Gadd45β by anti-diabetic drug Troglitazone (see Section 4.1) may point to the anti-diabetic potential of Gadd45 molecules. Indeed, an increased expression of Gadd45β may contribute to the amelioration of chronic insulin resistance, one of the major complications of diabetes. The underlying molecular mechanism could be linked to the Gadd45β-induced inhibition of TNFα cytotoxicity by suppressing TNFα-induced JNK signaling pathway, whose activation leads to chronic insulin resistance (Tuncman et al., 2006). Interestingly, insulin induces *Gadd45β* transcription by activating the mTOR pathway (Bortoff et al., 2010), well known for its association with aging, longevity, and ARDs (Blagosklonny, 2008; Zoncu et al., 2011).

There is direct and indirect evidence of the involvement of Gadd45 in major aging-associated conditions including oxidative stress, chronic inflammation, immunosenescence and fibroproliferative repair that, in turn, largely contribute to the development of ARDs and aging progression. The disbalance between pro- and anti-oxidant activities inclining towards elevated levels of oxidant species has long been considered a causative factor of aging. The age-related effects of oxidative stress on Gadd45 expression have not yet been fully addressed. It seems however that aging, at least in certain tissues, negatively affects the ability of cells to express Gadd45 proteins in response to oxidative stress. For example, when subjected to paraquat which promotes generation of superoxide radicals, cardiomyocytes of young mice increase expression of all three *Gadd45s*, whereas this does not occur in the myocardium of old animals (Edwards et al., 2004).

Another major condition underlying aging and ARDs is chronic inflammation which is largely attributed to an age-related increase in pro-inflammatory cytokines (TNFα, IL-1β,

IL-6, etc.) and NF-κB (Finch, 1990; Chung et al., 2009). In view of the links between these pro-inflammatory pathways and Gadd45s (described in Sections 3.3 and 3.6), the involvement of Gadd45 family in chronic inflammation is highly plausible. As an example, the induction of Gadd45 was observed in the course of liver inflammation (Gant et al., 2003). An additional source of the pro-inflammatory molecules are senescent cells which are accumulated in aging (Coppé et al., 2010). To what extent Gadd45s contribute to this process remains to be explored.

Both oxidative stress and chronic inflammation contribute to a process known as immunosenescence, in which the defects in T cells play a major role (Cannizzo et al., 2011; Larbi et al., 2011). Immunosenescence manifests in a decreased immune responsiveness to foreign and self-antigens, leading to an increased susceptibility to infection, cancer and autoimmune diseases. A decreased ability to maintain tolerance against self-antigens may result in autoimmune disorders. Mice with deficiency in *Gadd45β* and *Gadd45γ* spontaneously develop signs of autoimmune lymphoproliferative syndrome and systemic lupus erythematosus (Liu et al., 2005). In view of the key role of Gadd45β and Gadd45γ in maintaining the immunological tolerance (see Section 3.5), the reduced inducibility of Gadd45s in immune cells may stand behind an increased frequency of autoimmune conditions in aging (Liu et al., 2005).

One of the few examples where increased levels of Gadd45 may sustain the age-related immune dysfunctions is rheumatoid arthritis, whose incidence increases with advanced age (Lindstrom and Robinson, 2010). It is known that infiltrated Th1 cells in the synovial fluid of patients with rheumatoid arthritis are resistant to apoptosis. This resistance could be partially explained by the high levels of Gadd45β found in Th1 cells of synovial fluid and be primarily attributed to the stimulation of Gadd45β expression by pro-inflammatory cytokines TNFα and IL-12 (Du et al., 2008). As a result, the activated Th1cells avoid removal from the inflamed joints, thus contributing to chronic inflammation and tissue destruction. The important role of Gadd45β in this process also follows from the finding that silencing of Gadd45β by RNA interference abolished the anti-apoptotic effect of rheumatoid arthritis synovial fluid.

Pro-inflammatory cytokines and ROS, whose levels progressively increase in aging, may induce EMT, in particular, through activation of transcription factor NF-κB (López-Novoa and Nieto, 2009). In fact, EMT is the convergence point between inflammation, oxidative stress and the progression of degenerative fibrotic diseases and cancer. As mentioned above (see Section 4.1), Gadd45 closely cooperates with NF-κB and other EMT key players, β-catenin and MMPs, and by this way may thus contribute to EMT and associated pathologies.

4.3. Gadd45 protein partners and ARDs

It has been reported thus far that Gadd45s directly interact with 19 proteins (depicted in Fig. 1, Section 2). The links between Gadd45s and ARDs may to a great extent be realized through the interactions of Gadd45s with their first-order protein partners and regulators. As shown in Table 3, most of Gadd45 partners have been established as being strongly involved in at least one ARD (17 Gadd45 partners are involved in cancer, 8 in atherosclerosis, 7 in diabetes, and 6 in Alzheimer's disease), with several being involved in all or almost all major human ARDs. Important examples include the peroxisome proliferator-activated receptor family (PPARs) and CDKN1A (p21). The nuclear hormone receptor PPARγ has been implicated in the pathogenesis of numerous diseases including obesity, diabetes, atherosclerosis, Alzheimer's disease and cancer (Zieleniak et al., 2008; Schmidt et al., 2010). PPARγ is activated by thiazolidinediones (TZDs), insulin-sensitizing agents used for the treatment of type 2 diabetes. These drugs have also been shown to induce apoptosis in the vascular muscle cells through the upregulation of Gadd45 by activated PPARγ, and by

this way may reduce the formation of atherosclerotic lesions (Bruemmer et al., 2003). PPARγ possesses anti-aging and anti-inflammatory activities (Chung et al., 2008). To what extent they are mediated by Gadd45 remains a point for future investigations. The involvement of PPARγ in the regulation of NF-κB signaling (Cabrero et al., 2002), and interactions of Gadd45 with both of them strengthen this suggestion.

Another example is CDKNA1 (p21). This important regulator of cell cycle is deeply involved in cellular senescence, cancer, atherosclerosis, and diabetes (Chang et al., 2000). Its interaction with Gadd45 was shown to inhibit cell growth and as such, could be part of p21-mediated tumor suppressor activity (Zhao et al., 2000).

Apart from direct protein–protein interactions, there are numerous regulatory links between Gadd45 and well known players in ARDs, such as p53, p38, JNK, FOXO, SIRT1, HDAC, and others (see previous sections and Fig. 3).

4.4. GADD45 and longevity

As mentioned in previous sections, among the Gadd45 partners are several well-known longevity regulators. Furthermore, Gadd45 is among the targets of a number of established longevity-associated genes (LAGs). An example is the regulation of Gadd45 expression by two well established LAGs, SIRT1 and FOXO (see also Section 3). Depletion of SIRT1 expression by RNA interference results in a marked inhibition of FOXO-mediated Gadd45 induction in response to oxidative stress (Kobayashi et al., 2005).

The links between LAGs and Gadd45s make them highly probable candidates for the control of life span. In view of its functions (described in previous sections), it seems reasonable that over-expression of Gadd45 might promote longevity, in particular, by increasing the efficiency of DNA repair. Indeed, an opposite situation caused by mutations in the DNA repair genes may lead to hereditary syndromes of premature aging (e.g., Werner syndrome) (Navarro et al., 2006).

With the above in mind, we have recently developed the transgenic fruit flies overexpressing *dGadd45* in the nervous system, using the ELAV-Switch neuronal specific GAL4 driver (Plyusnina et al., 2011). By means of this model system, we have shown for the first time that the constitutive or conditional overexpression of *dGadd45* in the nervous system increases median and maximum Drosophila life span, without affecting fertility or physical activity. The important point is that the dGadd45-induced life span extension was accompanied by a considerable decrease (by approximately 25%) in the number of single-stranded breaks in DNA of Drosophila larvae neuroblast, as compared to their wild-type counterparts (Plyusnina et al., 2011). It should also be noted that in a wild-type fruit flies (Canton-S line), dGadd45 undergoes dramatic downregulation during the second half of life (our unpublished data). Altogether, these finding suggests that the longevity-promoting effect of Gadd45 overexpression could be causatively related to a more efficient detection and correction of spontaneous DNA damage.

5. Concluding remarks

Because of their pleiotropic action, a decreased inducibility of *Gadd45* members may have far-reaching consequences including genome instability, accumulation of DNA damage, and disorders in cellular homeostasis – all of which may eventually contribute to the aging process, promotion of tumorigenesis, immune disorders, insulin resistance and reduced responsiveness to stress (Fig. 4). Gadd45s stand at the crossroad of the cell fates by controlling the balance between cell (DNA) repair, eliminating (apoptosis) or preventing the expansion of potentially dangerous cells (cell cycle arrest, cellular senescence), and

maintaining the stem cell pool. Furthermore, Gadd45s are unique in coupling the DNA repair to epigenetic regulation. An additional and still unexplored possibility, with potential relevance to aging and ARDs, is that Gadd45s could be involved in the regulation of cell polarity. Gadd45s contain RNA-binding domains and are part of ribonucleoprotein particles (Sytnikova et al., 2011) which are often involved and play an important role in polar processes (Herr et al., 2008; Mili and Macara, 2009). Our analysis showed that RNA-binding proteins are a significantly enriched category among the polarity- and longevity-associated genes (Budovsky et al., 2011).

Despite the fact that the regulation of Gadd45s is much more complex in mammals than in lower organisms, our recent discovery that the dGadd45 gene is a longevity regulator in Drosophila (Plyusnina et al., 2011) offers hope that controlled manipulations of Gadd45s and its interacting partners may also bring benefits to humans. Remarkably, among the pro-longevity genes found in the lower model organisms that normally do not develop tumors, many are orthologous to human tumor suppressors (Budovsky et al., 2009). Given a strong association between cancer and aging, based on the common evolutionary and molecular mechanisms, it seems plausible that factors able to suppress cancer could also inhibit aging and *vice verse* (Blagosklonny, 2008). Thus, the anti-cancer activity of Gadd45s may presumably contribute to slowing or delaying the aging process. This could be also true with regard to another major human ARD – diabetes, as Gadd45β offers the potential of being a target for ameliorating the diabetes complications (Bortoff et al., 2010). Whether Gadd45s operate in the same manner in other ARDs and aging-associated conditions is still an unresolved issue and should be a point for future investigations.

It is important to note that Gadd45s are particularly suitable for manipulations as they are rapidly induced by various stressful stimuli. Furthermore, the moderate stimulation of cellular mechanisms of stress response often results in pro-longevity effects, even when being activated in the absence of the stress.

While Gadd45 family has been intensively studied, the biogerontological aspects have not as yet received a sufficient attention. Although further wide-scale research is warranted, it is becoming increasingly clear that Gadd45s are highly relevant to aging, ARDs and to the control of life span, suggesting them as potential therapeutic targets in ARDs and pro-longevity interventions.

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Appendix A. Construction and pathway analysis of GADD45-associated regulatory networks, with the focus on cell signaling and links to aging

To generate graphs of the Gadd45 family regulatory networks, we used the web-based bioinformatics software, Ingenuity Pathway Analysis (IPA, Ingenuity Systems, www.ingenuity.com). IPA is a curated database generated from more than 1.7 million published peer-reviewed scientific publications. Molecules are represented as nodes, and the biological relationship between two nodes is represented as an edge (line). All edges are supported by at least one reference from the literature or from canonical information stored in the Ingenuity Pathways Knowledge Base. Human, mouse, and rat orthologs of a gene are stored as separate objects in the Ingenuity Pathways Knowledge Base, but are represented as a single node in the network. Nodes are displayed using various shapes that represent the functional class of the gene product (see the legends for Figs. 2 and 3). The Path Designer module of IPA was used for creation of the graphs and cell art.

Using IPA software, we have designed graphical representations of the Gadd45-centered networks. These networks depict interactions of Gadd45 molecules with signal transduction and other intracellular regulatory pathways. In addition to IPA, we also used peer-reviewed literature to search for experimentally confirmed interactions of Gadd45s with small molecules, environmental and other factors that affect Gadd45 expression.

The constructed Gadd45α, β and γ interaction networks displayed 100, 88, and 88 direct molecular interactions, respectively. The majority of regulatory interactions of Gadd45 proteins are with transcription factors/regulators, small molecules (drugs or chemicals), cytokines, kinases, steroid hormone receptors, growth factors and miRNAs. Additionally, the following canonical IPA signal transduction pathways, associated with Gadd45s, have been represented: ATM Signaling; Hereditary Breast Cancer Signaling; Cell cycle: DNA Damage Checkpoint Regulation; p53 Signaling; Role of BRCA1 in DNA Damage Response; SAPK-JNK Signaling; and VDR-RXR Activation.

For the sake of simplicity, we labeled the central molecule of the graphs as “GADD45”, even though the molecular interactions represent all three GADD45 family members. In order to achieve the most optimal visual representation and avoid figure overcrowding, a selection process was applied to display only the key interactions for inducers of GADD45 (listed mainly on the top of the figures) or intracellular responders (listed mainly towards the bottom). Besides information available from IPA, we have also overlaid Fig. 3 with data concerning the age-related functional alterations, diseases and conditions.

Appendix B. The list of genes/proteins represented in Figs. 1–3 and Table 3

ABL1, c-abl oncogene 1; APC/APC2, adenomatous polyposis coli/adenomatosis polyposis coli 2; ATF2, activating transcription factor 2; ATM, ataxia telangiectasia mutated; ATR,

ataxia telangiectasia and Rad3 related; AXIN1, axin 1; BCL2, B-cell CLL/lymphoma2; BRCA1, breast cancer 1; CCNB1, cyclin B1; CDK1, cyclin-dependent kinase 1 (CDC2); CDKN1A, cyclin-dependent kinase inhibitor 1A (p21CIP1); CSF1, colony stimulating factor 1; CTNNB1, catenin (cadherin-associated protein), beta 1; Cyclin B; DCTN2, dynactin 2 (p50); ERCC5, excision repair cross-complementing rodent repair deficiency, complementation group 5 (XPG, XPGC); ERK, mitogen-activated protein kinase 1 (MAPK1); ESR1, estrogen receptor 1; FOXO1, forkhead box O1; GADD45, growth arrest and DNA-damage-inducible gene 45; GADD45GIP1, growth arrest and DNA-damage-inducible, gamma interacting protein 1; GM-CSF, granulocyte-macrophage colony-stimulating-factor; GRB2, growth factor receptor-bound protein 2; GRIN1, glutamate receptor, ionotropic, NMDA 1; GSK3B, glycogen synthase kinase 3 beta; GSK3B, glycogen synthase kinase 3 beta; IKBKG, inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase gamma; IL1R1, interleukin 1 receptor type I; IL3, interleukin 3; IL6, interleukin 6; Jnk, c-Jun NH2-terminal kinase; JUN, jun proto-oncogene; MAP2K7, mitogen-activated protein kinase kinase 7; MAP3K4, mitogen-activated protein kinase 4; MAP3K5, mitogen-activated protein kinase kinase kinase 5; MAPK14, mitogen-activated protein kinase 14; MRE11A, MRE11 meiotic recombination 11 homolog A; MYC, my proto-oncogene protein; NBN, nibrin; NBS1; Nijmegen breakage syndrome 1; NFAT5, nuclear factor of activated T-cells 5; NF-κB, nuclear factor kappa-B; NGF, nerve growth factor; NMDA Receptor, P38 MAPK, p38 mitogen-activated protein kinase; PCNA, proliferating cell nuclear antigen; PPARA, peroxisome proliferator-activated receptor alpha; PPARD, peroxisome proliferator-activated receptor delta; PPARG, peroxisome proliferator-activated receptor gamma; RAD50, DNA repair protein Rad50; RAD51, DNA repair protein Rad51; RARA, retinoic acid receptor, alpha; RWDD2B, RWD domain containing 2B; RXRA, retinoid X receptor, alpha; Sapk, mitogen-activated protein kinase 9 (Jun kinase, MAPK9); SMAD4, MAD homolog 4; STAT3, signal transducer and activator of transcription 3 (acute-phase response factor); STAT5A, signal transducer and activator of transcription 5A; SWI/SNF, matrix-associated actin-dependent chromatin remodeling complex; TFIID, transcription initiation factor TFIID; Tgf beta, transforming growth factor, beta; TGFBR2, transforming growth factor, beta receptor II; TNFRSF1A, tumor necrosis factor receptor superfamily, member 1A; TSNAX, translin-associated factor X; TP53, tumor suppressor protein p53; TP73, tumor protein p73; XPC, xeroderma pigmentosum, complementation group C.

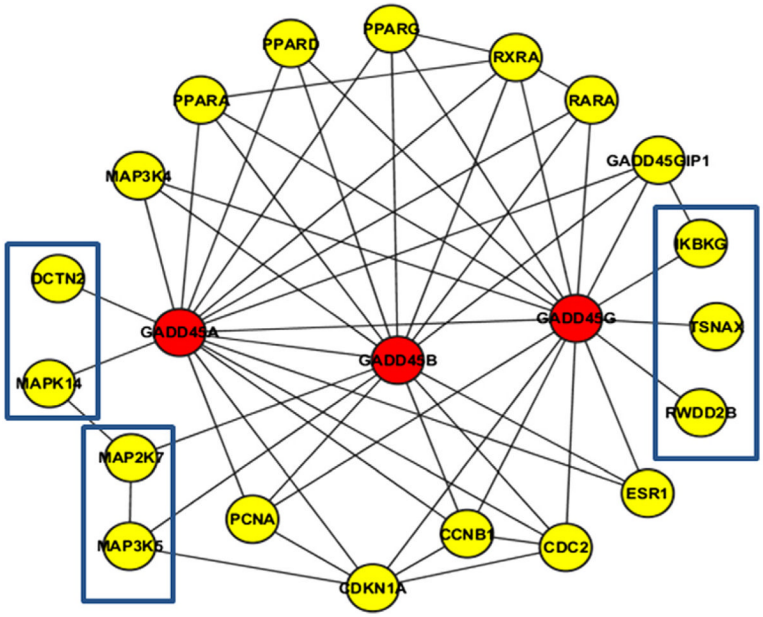

Fig. 1.

The protein–protein interaction network (PPI) of Gadd45α, β and γ with their first-order partners. The PPI data were extracted from the BioGRID database (Stark et al., 2011; http://thebiogrid.org), human interactome release 3.1.71, using the YABNA (Yet Another Biological Networks Analyzer) software program, previously described (Tacutu et al., 2010; http://www.netage-project.org). The graphical output was generated using Cytoscape 2.8.0 (Shannon et al., 2003; http://www.cytoscape.org/). Note that the vast majority of Gadd45 partners form connections with all three Gadd45 proteins. Nodes enclosed in rectangles – unique partners of Gadd45α, β or γ. For the details on gene/protein abbreviations, see Appendix B.

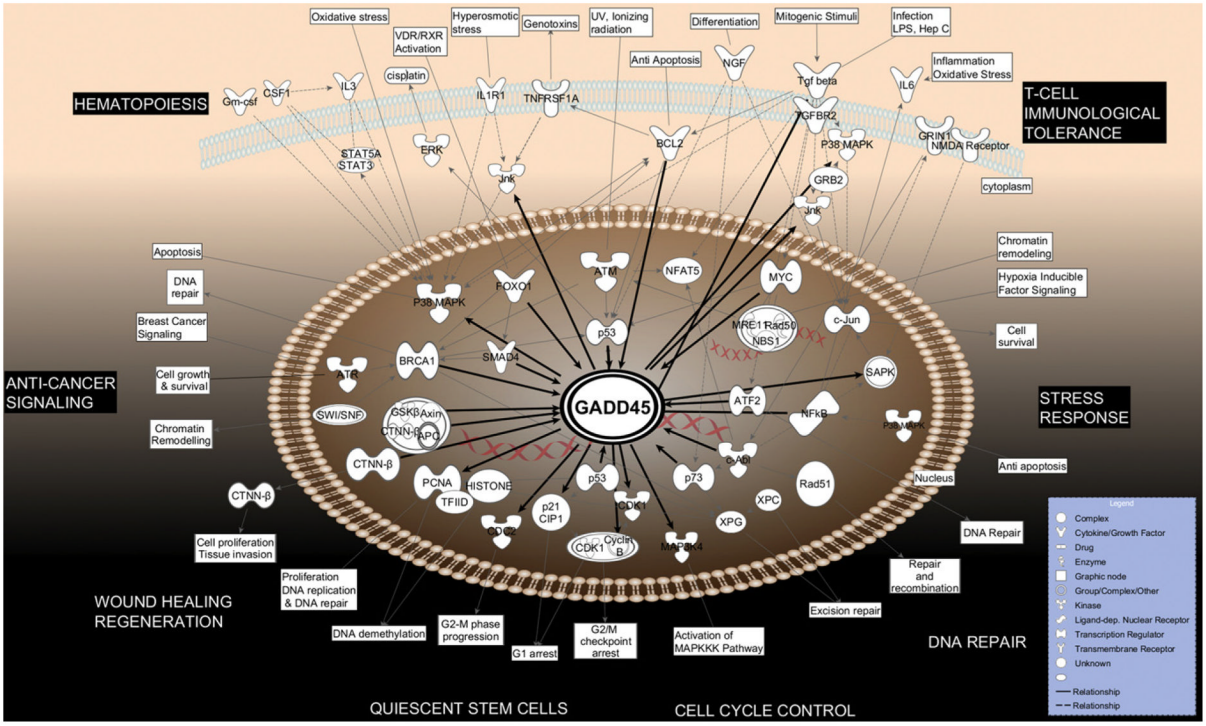

Fig. 2.

Map of molecular interactions involving GADD45 proteins and cell signaling pathways generated using Ingenuity Pathway Analysis software (IPA). Top functions identified by IPA were Cell Death, Cell Cycle and Cell Growth and Proliferation. Each of the node shapes denotes the function of the interacting protein or group of proteins. Molecular interactions are indicated by the lines between nodes. For the details on gene/protein abbreviations, see Appendix B.

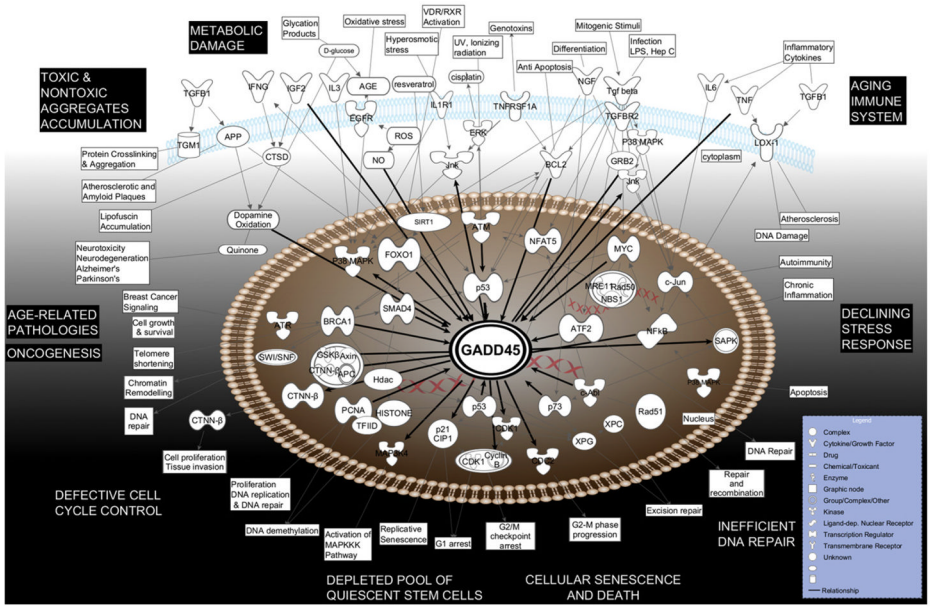

Fig. 3.

Map of molecular interactions of GADD45 proteins related to aging processes. Generated using Ingenuity Pathway Analysis software. Top functions and the node shapes as indicated in Fig. 2. For the details on gene/protein abbreviations, see Appendix B.

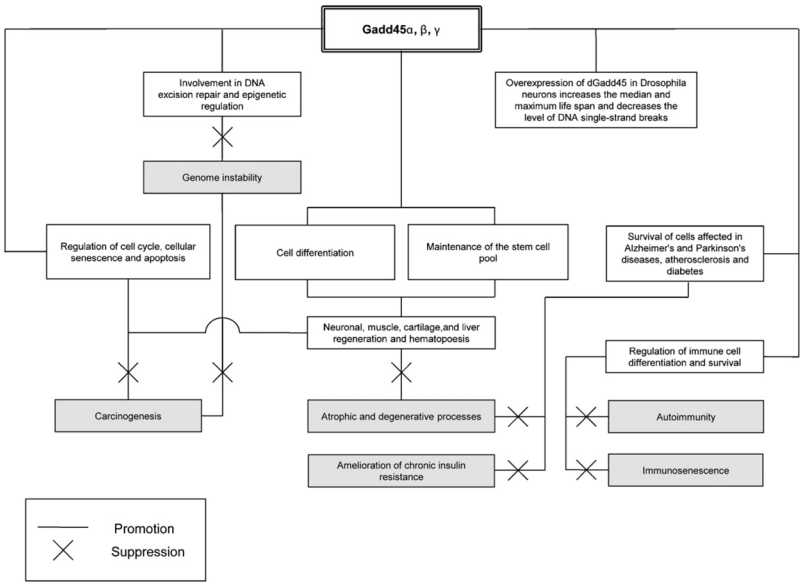

Fig. 4.
Summary scheme: the main anti-aging and pro-longevity activities of Gadd45 family.

Table 1

Induction of Gadd45 expression by various stresses.

Stress	Model	Gadd45 member	Reference
UV-radiation	Chinese hamster cells	Gadd45α	Fornace et al. (1988)
	HPV-immortalized oral keratinocytes	Gadd45α	Gujuluva et al. (1994)
	HPV-positive cervical cancer cell lines	Gadd45α	Butz et al. (1999)
	Human skin keratinocytes and human skin *in vivo*	Gadd45α	Wan et al. (2000)
	M1 myeloblastic leukemia and H1299 lung carcinoma cells	Gadd45α/β/γ	Zhang et al. (2001)
	Human keratinocytes	Gaddd45α Gadd45α/β	Maeda et al. (2003) Thyss et al. (2005)
	Human Caucasian melanocytes	Gaddd45α	Marrot et al. (2005)
X-rays	Human lymphoblast and fibroblast lines	Gadd45α	Papathanasiou et al. (1991)
	Rat seminiferous tubules	Gadd45α	West et al. (2002)
α-Radiation	HPV-positive cervical cancer cell lines	Gadd45α	Butz et al. (1999)
	Aortic endothelial cells	Gadd45α	Gajdusek et al. (2001)
	M1 myeloblastic leukemia and H1299 lung carcinoma cell lines	Gadd45α/β/γ	Zhang et al. (2001)
	ML-1 human myeloid tumor cell line	Gadd45α	Fornace et al. (2002)
Low-frequency electromagnetic fields	Mouse embryonic stem cells	Gadd45α	Nikolova et al. (2005)
Hypoxia	NIH-3T3 cells	Gadd45α	Price and Calderwood (1992)
	H460 human lung cancer cell line	Gadd45α	Corn and El-Deiry (2007)
Peroxynitrite free radicals	SH-SY5Y human neuroblastoma cells	Gadd45α	Oh-Hashi et al. (2001)
Hyperosmotic stress	Mouse inner medullary collecting duct cells	Gadd45α Gadd45α/β/γ	Kültz et al. (1998) Chakravarty et al. (2002)
	RKO cells	Gadd45α/β	Zumbrun et al. (2009)
	Mouse *NFAT5*$^{-/-}$ T lymphocytes	Gadd45α	Drews-Elger et al. (2009)
Oncogenic stress	Mouse embryo fibroblasts	Gadd45α	Bulavin et al. (2003)
Low pH	OE-33 oesophageal cells	Gadd45α	Duggan et al. (2006)
Delta12-prostaglandin J2	HeLa cells	Gadd45α	Ohtani-Fujita et al. (1998)
Component of the C5b-9 complement system	Rat podocytes *in vitro* and differentiated postmitotic mouse podocytes *in vivo*	Gadd45α	Pippin et al. (2003)
Arsenic As(III)	Offspring of DBA2 female with C57BL/6 male mice	Gadd45α	Simeonova et al. (2000)
	BEAS-2B human bronchial epithelial cell line	Gadd45α	Chen et al. (2001) Bower et al. (2006)
	Male 129/Sv mice	Gadd45α	Liu et al. (2001)
	MiaPaCa2 and PANC-1 cells	Gadd45α	Li et al. (2003)
	AsPC-1 human pancreatic, HT-29colonic, and MCF-7 breast cancer cells	Gadd45α	Li et al. (2004)
Sodium arsenite	CT7 mouse BAC clone	Gadd45β	Zhang et al. (2005)
Chromium Cr(VI)	Human lung fibroblasts	Gadd45α	Ceryak et al. (2004)
Cisplatin	Human ovarian cancer cell lines	Gadd45α	De Feudis et al. (1997) Delmastro et al. (1997)

Stress	Model	Gadd45 member	Reference
	HPV-positive cervical cancer cell lines	Gadd45α	Butz et al. (1999)
Methyl methanesulfonate	Human lymphoblast and human fibroblast lines	Gadd45α	Papathanasiou et al. (1991)
	M1 myeloblastic leukemia and H1299 lung carcinoma cell lines	Gadd45α/β/γ	Zhang et al. (2001)
Ethanol	Wild-type mice	Gadd45α	Ji et al. (2005)
Cigarette smoke condensate	MCF10A breast epithelial cell line	Gadd45α	Narayan et al. (2004)
	NHBE cells	Gadd45α	Fields et al. (2005)
Mitomycin C	HPV-positive cervical cancer cell lines	Gadd45α	Butz et al. (1999)
Bacterial lipopolysaccharides	CT7 mouse BAC clone	Gadd45β	Zhang et al. (2005)
	C57BL/6 male mice	Gadd45γ	Altemeier et al. (2005)
Pro-inflammatory cytokines IL-6, IL-12, and IL-18;	Th1cells	Gadd45β Gadd45β	Yang et al. (2001) Du et al. (2008)
IL-2 and IL-6	M1 mouse myeloid leukemia cell line and M1p53 cell line	Gadd45γ	Zhang et al. (1999)
Pro-apoptotic cytokines TGFβ and TNFα	M1 myeloblastic leukemic cells	Gadd45β	Selvakumaran et al. (1994)
	HeLa, COS-7 and C2C12 cells	Gadd45β	Takekawa et al. (2002)
	AML12 mouse hepatocytes	Gadd45β	Yoo et al. (2003)
	Th1 cells	Gadd45β	Du et al. (2008)
	CT7 mouse BAC clone	Gadd45β	Zhang et al. (2005)

Table 2

Phenotypic characteristics of Gadd45 knockout mice.

Targeted Gadd45 member	Observed phenotype	Reference
Gadd45α$^{-/-}$	Genomic instability, abnormal nucleotide- and base-excision DNA repair, increased sensitivity to carcinogenesis	Hollander et al. (1999, 2001), Tront et al. (2006), Maeda et al. (2005)
	Impaired ability for cell differentiation and increased sensitivity to the induction of apoptosis	Gupta et al. (2006)
	Increased sensitivity to skin irradiation	Hildesheim et al. (2002)
	Premature death, auto-immunity	Salvador et al. (2002)
	Abnormal hematopoiesis	Salvador et al. (2002), Gupta et al. (2005)
	Depletion of stem cell pool; decreased clonogenic potential of myeloid progenitors	Hoffman and Liebermann (2007)
	Nervous, reproductive and renal disorders	Hollander et al. (1999), Salvador et al. (2002)
Double KO: Gadd45α$^{-/-}$, XPC$^{-/-}$	Premature death and increased tumorigenesis	Hollander et al. (2005)
Double KO: Gadd45α$^{-/-}$, Cdkn1a$^{-/-}$	Premature death, auto-immunity, abnormal hematopoiesis, impaired homeostasis, renal disorders	Salvador et al. (2002)
	Abnormal chromosome number, increased cell proliferation	Hollander et al. (1999)
	Decreased cellular sensitivity to ultraviolet irradiation, normal nucleotide-excision repair	Maeda et al. (2005)
Double KO: Gadd45α$^{-/-}$, Brca1$^{-/-}$	Exencephaly at embryonic days 9.5–10.5, complete prenatal lethality	Wang et al. (2004)
Gadd45β$^{-/-}$	Impaired immunity and anti-tumor immune response; increased susceptibility to ionizing radiation and chemical carcinogens	Ju et al. (2009), Lu et al. (2004)
	Autoimmune conditions	Liu et al. (2005)
	Impaired ability for differentiation and increased sensitivity to the induction of apoptosis	Gupta et al. (2006)
	Abnormal hematopoiesis	Gupta et al. (2005)
	Depletion of stem cell pool; decreased clonogenic potential of myeloid progenitors	Hoffman and Liebermann (2007)
Gadd45γ$^{-/-}$	Impaired immunity	Lu et al. (2001), Lu et al. (2004)
	Normal hematopoiesis and proliferative response to IL-2	Hoffmeyer et al. (2001)
	Abnormal cell physiology	Cai et al. (2006)
Double KO: Gadd45β$^{-/-}$, Gadd45γ$^{-/-}$	Impaired immunity and anti-tumor immune response; Autoimmune conditions	Ju et al. (2009), Lu et al. (2004) Liu et al. (2005)
Triple KO: Gadd45α$^{-/-}$, Gadd45β$^{-/-}$, Gadd45γ$^{-/-}$	Increased cell proliferation in the renal outer medullary cells	Cai et al. (2006)
Ras-over expressing mice: Gadd45α$^{+/-}$ or Gadd45α$^{-/-}$	Ras-driven breast tumorigenesis is Gadd45α-dependent; Gadd45α inhibits the onset and growth of mammary tumors	Tront et al. (2006)

Table 3

The involvement of Gadd45 direct proteins partners in age-related diseases.

Gene[a]	Cancer	Atherosclerosis	Diabetes	AD[b]	LAGs[c]
CCNB1	+				
CDC2	+	+			
CDKN1A	+	+	+	+	
DCTN2	+				
ESR1	+	+	+		+
GADD45GIP1	+				
IKBKG	+				
MAP2K7	+				
MAP3K4		+			
MAP3K5	+	+			
MAPK14	+	+	+	+	
PCNA	+	+			
PPARA	+	+	+	+	
PPARD	+	+	+		
PPARG	+	+	+	+	+
RARA	+				
RWDD2B	+				
RXRA	+	+			
TSNAX					

[a] For the details on gene/protein abbreviations, see Appendix B.

[b] Alzheimer's disease.

[c] Longevity-associated genes were extracted from http://genomics.senescence.info/species/ (de Magalhaes et al., 2009) and http://netage-project.org/ (Tacutu et al., 2010).